

advantage is that the response of platelets to ADP (in the presence of added fibrinogen) remains similar to that in native plasma. Aggregation in response to ADP, without thromboxane production or release of granule contents, is the characteristic of a good preparation.

[2] Isolation of Human Platelets by Albumin Gradient and Gel Filtration

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Human blood platelets interact with several plasma proteins that participate in the formation of a hemostatic plug.¹ To study the interactions of plasma proteins with their receptors on the platelet membrane a preparation of platelets free of plasma proteins is needed in order to assure that they will not interfere with the binding of labeled ligands. A number of methods provide platelets that have been used for testing the effect of plasma proteins and general requirements have been formulated.² The methods which employ repeated washing and centrifugation work best when a relatively large volume of blood is used.³ Isolation of platelets from small samples of blood, i.e., 20–40 ml, by repeated washing and centrifugation usually ends with a poor yield of platelets. In some instances washed platelets are treated with formalin for agglutination studies.⁴ Such treated platelets cannot be used for studying processes requiring metabolically intact platelets, e.g., signal transduction generated by ADP, epinephrine, and arachidonic acid.

The second group of methods of separation of platelets from plasma proteins includes gel filtration and/or centrifugation over a cushion or gradient made of albumin, Ficoll, or stractan.^{5–12} The gel filtration method

¹ J. Hawiger, in "Hemostasis and Thrombosis: Basic Principles and Clinical Practice" (R. Colman, J. Hirsh, V. Marder, and E. Salzman, eds.), 2nd Ed., p. 162. Lippincott, Philadelphia, Pennsylvania, 1987.

² H. J. Day, H. Holmsen, and M. B. Zucker, *Thromb. Diath. Haemorrh.* **33**, 648 (1975).

³ J. F. Mustard, D. W. Perry, N. H. Ardlie, and M. A. Packham, *Br. J. Haematol.* **22**, 193 (1972).

⁴ D. E. Macfarlane, J. Stibbe, E. P. Kirby, and M. B. Zucker, *Thromb. Diath. Haemorrh.* **34**, 306 (1975).

⁵ S. Timmons and J. Hawiger, *Thromb. Res.* **12**, 298 (1978).

introduced by Tangen and colleagues⁶ posed some difficulties due to inadvertent activation of platelets, dilution of the platelet suspension, and the use of Tris buffer, which affects platelet function. Subsequent modification of this method improved the quality of the platelet preparation.⁹ However, gel-filtered platelets retain a residual quantity of large plasma proteins, such as von Willebrand factor (vWF) and fibrinogen, which may influence the study of platelet receptors for these adhesive macromolecules.⁵ A similar problem concerns platelet preparation on an albumin cushion or gradient, originally developed by Walsh.⁷ We have adapted these methods of platelet separation from plasma proteins by gel filtration and albumin gradient centrifugation to produce stable, functionally unimpaired platelets which are relatively free of absorbed plasma proteins, including the largest ones.⁵ The combined albumin gradient-gel filtration method described herein yields human platelets which are separated from plasma proteins, including vWF and fibrinogen, as judged by a negative response to ristocetin and ADP, respectively. The stability of the obtained platelet preparation has been enhanced by our application of HEPES buffer^{5,13} which, in contrast to Tyrode's buffer, is not affected by changes in the $p\text{CO}_2$ of the platelet suspension. Subsequent to our introduction of HEPES buffer to platelet isolation,⁵ other workers have since employed HEPES buffer in their experiments with platelets.^{14,15} The use of Tris buffer in platelet work is not recommended due to its detrimental effect on calcium fluxes.¹⁶

Materials and Reagents

Preparation of 50% Albumin. Deionized water (200 ml) is poured in a 1-liter Erlenmeyer flask, followed by 100 g of bovine albumin (fraction V, reagent grade, Miles Laboratories, Naperville, IL). The mixture should

⁶ O. Tangen, H. J. Berman, and P. Marfey, *Thromb. Diath. Haemorrh.* **25**, 268 (1971).

⁷ P. M. Walsh, *Br. J. Haematol.* **22**, 205 (1972).

⁸ R. A. Hutton, M. A. Howard, D. Deykin, and R. M. Hardisty, *Thromb. Diath. Haemorrh.* **31**, 119 (1974).

⁹ B. Lages, M. C. Scrutton, and H. Holmsen, *J. Lab. Clin. Med.* **85**, 811 (1975).

¹⁰ D. G. Nichols and J. R. Hampton, *Thromb. Diath. Haemorrh.* **27**, 425 (1972).

¹¹ L. Corash, B. Shafer, and M. Perlow, *Blood* **52**, 726 (1978).

¹² P. Ganguly and W. J. Sonnichsen, *J. Clin. Pathol.* **26**, 635 (1973).

¹³ C. Shipman, Jr., *Proc. Soc. Exp. Med. Biol.* **130**, 305 (1969).

¹⁴ E. I. Peerschke, M. B. Zuker, R. A. Grant, J. J. Egan, and M. M. Johnson, *Blood* **55**, 841 (1980).

¹⁵ Z. M. Ruggeri, L. De Marco, L. Gatti, R. Bader, and R. R. Montgomery, *J. Clin. Invest.* **72**, 1 (1983).

¹⁶ M. A. Packham, M. A. Gucionne, M. Nina, R. L. Kinlough-Rathbone, and J. F. Mustard, *Thromb. Haemostasis* **51**, 140 (1984).

remain unperturbed at 4° for 24–48 hr; do not mix until all albumin is dissolved. Since the pH of the dissolved albumin should be between 6.5 and 7.0, one should purchase reagent with specified pH of about 7 in solution. Aliquots (7 ml) are stored at –40° until needed. Then the 50% albumin is thawed and diluted with either HEPES buffer or Ca²⁺-free Tyrode's buffer (see below) to prepare 25, 17, 12, and 10% solutions. If the platelets are used for studies to examine the effects of ADP, apyrase (0.1 unit/ml) can be added to platelet-rich plasma (PRP) and the albumin solutions employed for preparation of the gradient.¹⁷ We found that addition of apyrase (EC 3.6.1.5) was helpful in assuring the sustained response of platelets to ADP when the common receptor mechanism for binding of fibrinogen and vWF to human platelets was studied.¹⁷

Purification of Apyrase. Commercially available apyrase (grade I) from Sigma is further purified by the method of Traverso-Cori *et al.*¹⁸ Briefly, 6000 units of apyrase is dissolved in 20 ml of 0.05 M potassium succinate in 0.3 M KCl buffer, pH 4.0, centrifuged at 10,000 g for 10 min, and desalted by passing through a Sephadex G-25 column (50 × 4 cm) that is equilibrated with the potassium succinate buffer, pH 4.0. The peak fractions are pooled, concentrated to 10 ml using an Amicon ultrafiltration unit with a PM30 filter, and then passed through a Sephadex G-150 column (80 × 2.5 cm) equilibrated with 0.05 M potassium succinate buffer in 0.3 M KCl. The fractions are assayed for protein at 280 nm and for ATPase activity. The unit of enzyme activity is that amount of enzyme that releases 1 μmol P_i/min.¹⁹ The fractions containing the highest ATPase activity per milligram of protein are pooled, dialyzed against phosphate-buffered saline, and concentrated to 10 ml on an Amicon ultrafiltration unit. Note that Sigma currently has available apyrase (grade VIII) of similar specific activity (300–400 units/mg of protein) which may be used.

Preparation of Gel Columns. Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ) or BioGel A-150, 100–200 mesh (Bio-Rad Laboratories, Richmond, CA) is prepared by acetone washing (3–4 vol) followed by thorough rinsing with 5–6 vol of 0.9% NaCl. Sepharose 2B prepared in this way may be refrigerated and used within 2 weeks. On the day of an experiment the Sepharose 2B gel suspended in 0.9% NaCl is warmed to room temperature, if necessary deaerated in a vacuum flask, and poured into 2 × 8 cm columns made of glass syringes. The inner bottom of the

¹⁷ S. Timmons, M. Kloczewiak, and J. Hawiger, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4935 (1984).

¹⁸ A. Traverso-Cori, S. Traverso, and H. Reyes, *Arch. Biochem. Biophys.* **137**, 133 (1970).

¹⁹ A. Traverso-Cori, H. Chaimovich, and O. Cori, *Arch. Biochem. Biophys.* **109**, 173 (1965).

syringe contains a disk of nylon microfilament with mesh opening of $52\ \mu\text{m}$ (Small Parts, Inc., Miami, FL) which is held in place by a plastic ring. The gel should be poured in such a way that air bubbles are not formed. The flow is regulated by a Teflon two-way stopcock to which polyethylene tubing is attached. All parts of the column are siliconized (Sigmacote, Sigma, St. Louis, MO), rinsed, and dried before first use. Before applying the platelet suspension, the gel columns are equilibrated with 100 ml of buffer as specified below. If air bubbles appear, the gel columns are repacked and then washed again.

Buffers. Two buffers for separation of platelets from plasma proteins can be used: HEPES (N-2-hydroxyethyl-1-piperazine-N'-2-ethanesulfonic acid)⁵ or Ca^{2+} -free Tyrode.³ HEPES buffer contains a final concentration of 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 5.5 mM glucose, 0.35% albumin, 3 mM NaH_2PO_4 , and 3.5 mM HEPES (Ultrol Grade, Calbiochem, La Jolla, CA). Tyrode's buffer contains a final concentration of 137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO_3 , 1 mM MgCl_2 , 5.5 mM Glucose, and 0.35% bovine albumin. The salts are prepared as 50 \times concentrated stock solutions. On the day of use the concentrated stock solutions are diluted, glucose and bovine albumin are dissolved into the buffer solution, and the pH adjusted to 7.35 with 1 N NaOH or 1 N HCl. The same buffer is used to dilute the albumin for gradients, to equilibrate the columns, and to elute the platelets therefrom.

Gel Filtration Method. Polypropylene plasticware or siliconized glassware should be used throughout the procedure. Blood from healthy volunteers, who have not ingested any aspirin for 10 days and who have not taken any other medication for at least 2 days, is collected into 0.12 M citrate buffer, pH 6.0 (0.01 M citric acid in 0.11 M sodium citrate). Nine parts blood is drawn into a syringe containing one part citrate buffer and centrifuged at 160 g for 15 min to obtain platelet-rich plasma (PRP). After collecting PRP, the remaining portion of blood is centrifuged at 3000 g for 10 min to obtain platelet-free plasma (PFP). The columns containing Sepharose 2B or BioGel A-150 are equilibrated with either HEPES buffer or Ca^{2+} -free Tyrode's buffer and are adjusted to have a flow rate of 1 ml/min. When the equilibrating buffer has just entered the gel, 2.5 ml PRP is applied directly to the top of the gel, avoiding disturbance of the interface. The platelets are eluted as indicated below. Because platelet suspensions prepared by single-step gel filtration contain plasma proteins, such as vWF and fibrinogen, modifications were introduced for a two-step procedure combining albumin cushion/gradient centrifugation and gel filtration.⁵

Combined Albumin Cushion/Gradient and Gel Filtration Method. The procedures are outlined diagrammatically in Fig. 1.

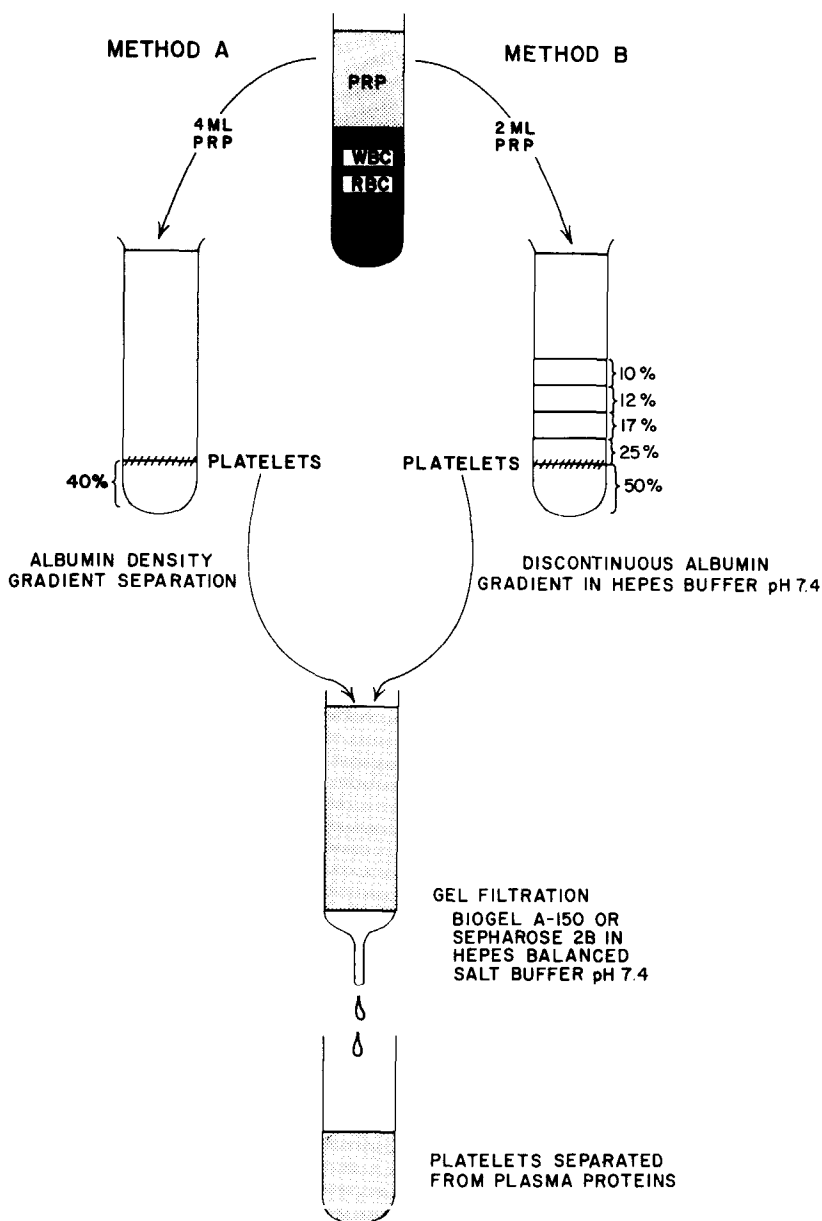


FIG. 1. Diagram outlining methods A and B of platelet isolation from plasma proteins. (Thromb. Res., Vol. 12, Timmons and Hawiger, "Separation of Platelets from Plasma Proteins Including Factor VIII_{vWF} by a Combined Albumin Gradient-Gel Filtration Method Using Hepes Buffer," 1978, Pergamon Journals, Ltd.)

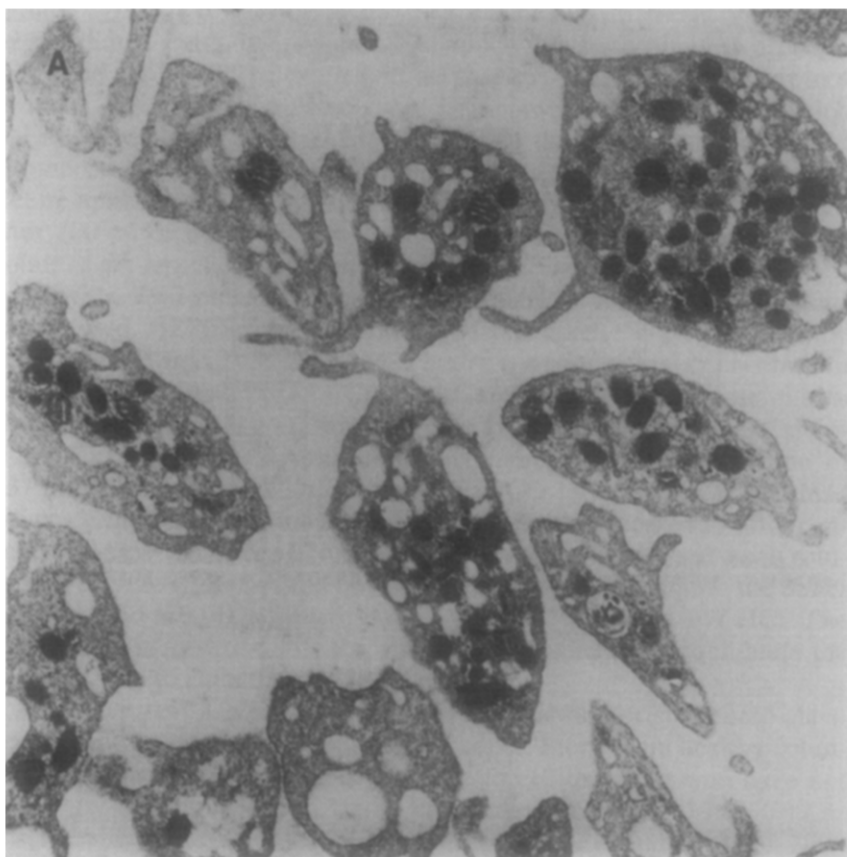


FIG. 2. Electron micrographs of (A) platelets in plasma and (B) platelets separated by the stepwise albumin gradient followed by Sepharose 2B gel filtration. $\times 32,400$. (Thromb. Res., Vol. 12, Timmons and Hawiger, "Separation of Platelets from Plasma Proteins Including Factor VIII_{WF} by a Combined Albumin Gradient-Gel Filtration Method Using Hepes Buffer," Pergamon Journals, Ltd.)

Method A: PRP (4 ml) is applied to a 50% albumin cushion⁷ and centrifuged at 1200 *g* for 15 min. The plasma is removed and, using another pipet, the platelet fraction resting on the top of the albumin cushion is collected, diluted in buffer to 2.5 ml, and applied to either a Sepharose 2B or BioGel A-150 column. Platelets are eluted with the same buffer which was used to equilibrate the column. The opaque fractions in the effluent containing platelets are collected, their absorbance determined at 520 nm, and only the fractions constituting the peak of the elution

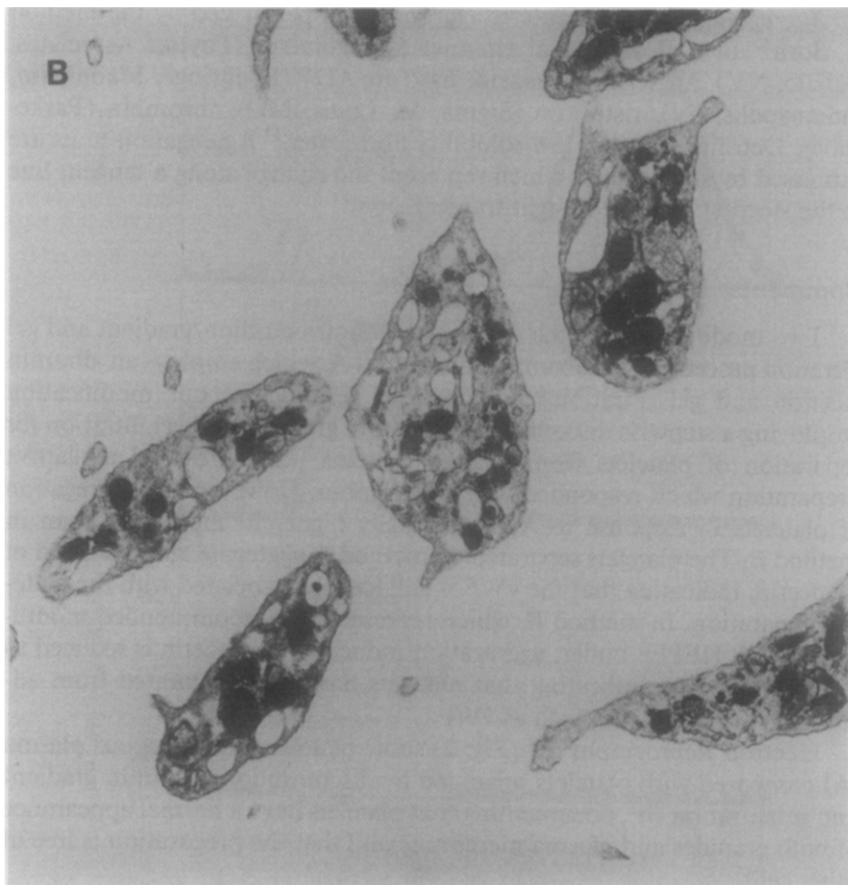


FIG. 2. (continued)

profile are pooled for platelet studies. The after-peak fractions of platelets, which are contaminated with vWF, should be discarded.

Method B: PRP (2 ml) is applied to a step-wise discontinuous albumin gradient (50, 25, 17, 12, 10%) and centrifuged at 1200 g for 15 min. The supernatant is removed with a Pasteur pipet, being careful to collect from the top of the fluid, and with another siliconized Pasteur pipet the platelet fraction is collected. The platelet fractions from three gradients are diluted in buffer to 2.5 ml, applied to either a Sepharose 2B or BioGel A-150 column, and platelets are eluted as described under method A.

Platelet Counts. These are performed on a Coulter electronic particle counter (Coulter Electronics, Hialeah, FL).

Aggregation Studies. Platelet aggregation is performed by the method of Born²⁰ in a Payton dual channel aggregometer (Payton Associates, Buffalo, NY). Aggregating reagents used are ADP (Boehringer Mannheim, Indianapolis, IN), ristocetin (Sigma, St. Louis, MO), thrombin (Parke-Davis, Detroit, MI), and low-solubility fibrinogen.¹⁷ Aggregation units are expressed by slope values which represent the change along a tangent line to the steepest increase in light transmission.²¹

Comments

Two modifications of the combined albumin cushion/gradient and gel filtration procedure were compared: method A, which employs an albumin cushion and gel filtration,⁸ and method B, which is our modification employing a stepwise discontinuous albumin gradient and gel filtration for separation of platelets from plasma proteins,⁵ and provided a platelet preparation which responded well to thrombin. However, the aggregation of platelets in response to ADP is usually higher in method A than in method B. The platelets separated by method A aggregate after addition of ristocetin, indicating that the vWF is still loosely associated with the platelet preparation. In method B, which represents the recommended modification with HEPES buffer, aggregation induced by ristocetin is reduced to a negligible level, indicating that platelets have been separated from adsorbed plasma proteins such as vWF.

Electron micrographs^{22,23} (Fig. 2) show platelets in autologous plasma (A) compared with platelets separated by discontinuous albumin gradient and gel filtration (B), documenting that platelets have a normal appearance of both granules and plasma membrane and that the preparation is free of other cells.

Although BioGel A-150 has a higher exclusion limit in comparison with Sepharose 2B the elution profiles from both gels are the same in terms of platelet count, absorbance at 520 nm, and protein content determined by absorbance at 280 nm (Fig. 3).

When the response of platelets to aggregating agents is compared in two buffer systems, the HEPES balanced salt solution introduced by us for platelet work⁵ and the conventional Tyrode's buffer, the aggregation response of platelets to ADP and epinephrine is similar during the first hour.

²⁰ G. V. R. Born, *Nature (London)* **194**, 927 (1962).

²¹ C. S. P. Jenkins, D. Meyer, M. D. Dreyfus, and M. J. Larrieu, *Br. J. Haematol.* **28**, 561 (1974).

²² G. Millonig, *J. Appl. Physiol.* **32**, 1637 (1961).

²³ E. S. Reynolds, *J. Cell Biol.* **17**, 208 (1963).

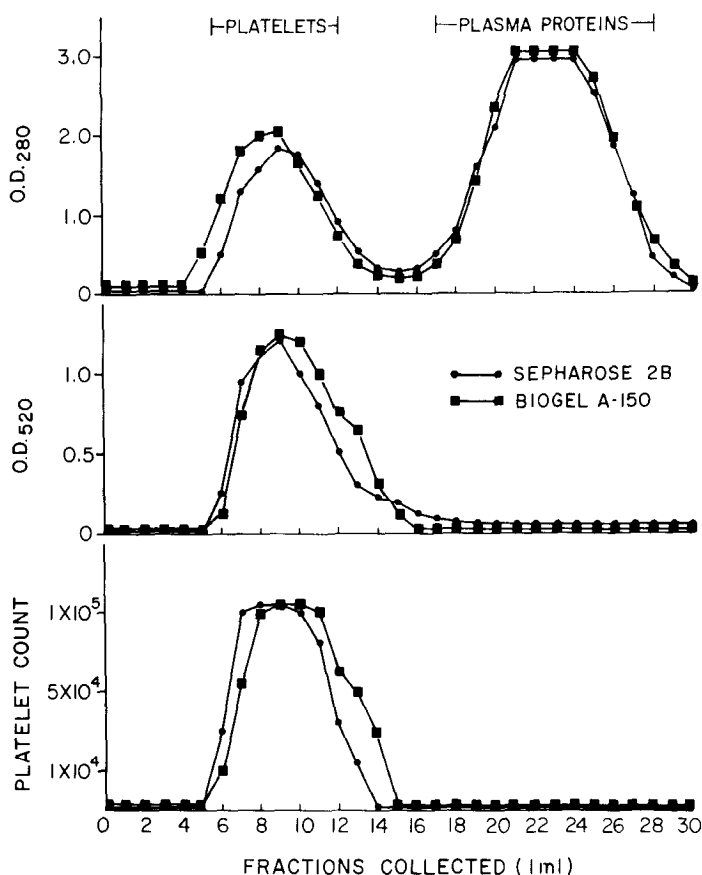


FIG. 3. Elution profiles comparing the patterns obtained when platelets are gel filtered through Sepharose 2B (●) or BioGel A-150 (■). (Thromb. Res., Vol. 12, Timmons and Hawiger, "Separation of Platelets from Plasma Proteins Including Factor VIII_{vWF} by a Combined Albumin Gradient-Gel Filtration Method Using Hepes Buffer," 1978, Pergamon Journals, Ltd.)

However, when aggregation is measured over a longer period of time (2 hr), the platelets separated and suspended in HEPES buffer maintain their responsiveness longer than the platelets suspended in Tyrode's buffer (Table I). It is noteworthy that the pH of the platelet suspension in HEPES buffer remains constant whereas the pH of the platelet suspension in Tyrode's buffer changes significantly upward due to the escape of CO₂.

Although the one-step gel filtration method is quick and simple, under ordinary conditions it does not separate platelets from the vWF protein and trace concentrations of fibrinogen. Changing the matrix to BioGel

TABLE I
EFFECT OF CONDITIONS AND TIME ON *in Vitro*
RESPONSIVENESS TO AGGREGATING AGENTS OF
PLATELETS SEPARATED BY METHOD B

| Condition | Time (hr) | | | |
|--------------------------------------------------------|-----------|-----|-----|-----|
| | 0 | 1 | 2 | 3 |
| Tyrode's buffer | | | | |
| pH | 7.4 | 7.7 | 8.0 | 8.0 |
| Aggregation ^a with ADP + Fbg ^b | 10 | 8 | 0 | 0 |
| Aggregation ^a with epinephrine ^c | 4 | 2 | 0 | 0 |
| Hepes buffer | | | | |
| pH | 7.4 | 7.4 | 7.4 | 7.4 |
| Aggregation ^a with ADP + Fbg ^b | 11.5 | 10 | 6.5 | 0 |
| Aggregation ^b with epinephrine ^c | 4 | 4 | 4 | 0 |

^a Aggregation units expressed as slope value obtained by drawing a tangent line to the steepest rise in the curve and calculating the change per 1 min.

^b ADP added to have a final concentration of 5 μ M and fibrinogen final concentration of 1.5 mg/ml.

^c Epinephrine added to have a final concentration of 50 μ M.

(From Thromb. Res., Vol. 12, Timmons and Howiger, "Separation of Platelets from Plasma Proteins Including Factor VIII_{vWF} by a Combined Albumin Gradient-Gel Filtration Method Using Hepes Buffer," 1978, Pergamon Journals, Ltd.)

A-150, which has a higher exclusion limit than currently used Sepharose 2B, does not improve separation. Platelets separated in this way still aggregate with ristocetin without addition of plasma. In the two-step procedure tested (method A) the platelet-rich plasma is first applied to an albumin cushion and then to a gel column.⁸ This has the advantage that platelets can be concentrated by reducing the volume of the resuspending buffer, but it does not separate platelets from plasma vWF as documented by a positive aggregation response to ristocetin. Finally, the use of a stepwise discontinuous albumin gradient prior to gel filtration results in a simple two-step method for separation of platelets from adsorbed plasma proteins including vWF. Platelets obtained through this procedure do not respond to ristocetin unless plasma as a source of vWF is added. Another modification of this procedure involves washing of platelets by centrifugation and resuspending the pellets in buffer before applying to a Sepharose 2B column.²⁴

²⁴ G. A. Marguerie, T. S. Edgington, and E. F. Plow, *J. Biol. Chem.* **255**, 154 (1980).

The use of HEPES buffer, instead of Tyrode's buffer, provides conditions for a preparation of platelets that is more stable and reactive for a substantially longer period of time. The zwitterionic buffer (HEPES) has been developed for biologic systems²⁵ and has proved satisfactory as a tissue culture buffer.¹³ It has a pK of 7.31, allowing the pH to remain constant for several hours. In contrast, commonly used Tyrode's buffer is prone to variation in CO_2 content of the platelet suspension and necessitates a variety of maneuvers to maintain the same level of CO_2 .²⁶ By the use of HEPES buffer the need for control of CO_2 is obviated.

Platelets prepared by method B do not become reactive to ristocetin in the absence of plasma for at least 3 hr, indicating that under these conditions they are not "leaking" vWF protein which has been found to be associated with subcellular membranes and granules.²⁷ Thus, the system described here as method B is useful for the quantitative study of the interaction of plasma proteins with blood platelets requiring careful control of the environment of the intact, metabolically active platelets.^{17,28} A similar procedure of platelet separation has been employed for studies of Na^+/H^+ exchange by using Na^+ -free buffer.²⁹

²⁵ N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and N. M. Singh, *Biochemistry* **5**, 467 (1966).

²⁶ A. B. Rogers, *Proc. Soc. Exp. Biol. Med.* **139**, 1100 (1972).

²⁷ R. L. Nachman and E. A. Jaffe, *J. Exp. Med.* **141**, 1101 (1975).

²⁸ T. Fujimoto, S. Ohara, and J. Hawiger, *J. Clin. Invest.* **69**, 1212 (1982).

²⁹ T. M. Connolly and L. E. Limbird, *J. Biol. Chem.* **258**, 3907 (1983).